

Method for In Vitro Diagnosis of Endometriosis

This application claims the benefit of the filing date of U.S. Provisional Application Serial No. 60/243,265 filed October 26, 2000.

The invention relates to a method for in vitro diagnosis of endometriosis.

Endometriosis is one of the most frequently occurring gynecological diseases, by which roughly 5-10% of all women of child-bearing age are affected (Sillem, M. 1998; Programmed^(R) 23, Suppl. 1, 1-28). It is characterized by the occurrence of endometrial tissue outside of the physiological mucous membrane lining of the uterus. In addition to pain and numerous other symptoms, many endometriosis patients are sterile, and a large portion of IVF patients (IVF = in vitro fertilization) suffer from endometriosis (Adamson, G. D. 1997; Sem. Reprod. Biol. 15, 263-271). Very recently, publications that speak for a genetic predisposition in the development of an endometriosis have been multiplying (Kennedy, S. 1997; Sem. Reprod. Biol. 15, 309-318). The loss of tumor suppressor molecules and family clusters in the case of endometriosis patients was thus described.

Endometriosis is currently diagnosed with the aid of laparoscopy. This is an invasive method, which frequently results in complications (Chapron, C. et al. 1998; Hum. Reprod. 13, 867-872; Jansen, F. W. et al., 1997; Br. J. Obstet. Gynecol. 104, 595-600). It is performed under anesthesia and requires a fully set-up operating room.

There is therefore a need for new diagnostic methods. A method that would impose less of a burden for the patients and that could be performed by the attending physician would be desirable.

This problem is achieved according to the invention by the identification of genes that are differentially regulated in endometriosis and the preparation of a method for detection of their gene products.

The invention relates to a method for in vitro diagnosis of endometriosis, whereby the amount of gene product of at least one gene from the group that consists of fibronectin, insulin-like growth factor binding protein-2, transmembrane receptor PTK7, platelet-derived growth factor receptor alpha, collagen type XVIII alpha 1, subtilisin-like protein (PACE4), laminin M chain (merosin), elastin, collagen type IV alpha 2, p27 interferon alpha-inducible gene, reticulocalbin, aldehyde dehydrogenase 6, gravin, nidogen and phospholipase C epsilon is determined in a patient sample and is compared to the amount of this gene product in a control sample, whereby a smaller amount of this gene product indicates the presence of an endometriosis.

The group of genes is described in more detail in Figure 1. The expression strength, i.e., the amount of gene product, is determined by at least one of the genes in a patient sample that is mentioned in Figure 1 and compared to that of a control sample (women without endometriosis). The samples that are to be compared must both originate from the secretory phase, thus from the range of days 15-28 after the last menstruation. A decreased

expression strength of at least one of the above-mentioned genes in the patient sample indicates the presence of an endometriosis.

A patient sample can be a sample from endometrial tissue, peritoneal fluid, blood, vaginal secretion or urine of the patient.

A gene product is either mRNA, the cDNA that is derived therefrom, a polypeptide or portions of a polypeptide. The amino acid sequences of the polypeptides are depicted in Figure 2.

The methods according to the invention can be used for first-time diagnosis of endometriosis. In this case, the amount of the gene product in the patient sample is compared to a control sample of undiseased women. The method according to the invention can also be used to evaluate the course of the disease. Thus, e.g., the success of a therapy can be determined. In this case, the patient sample is compared to a prior sample from the same patient.

The gene product polypeptide or a segment of a polypeptide is detected by immunoassay. To this end, specific antibodies are produced using one or more polypeptides that are selected from the group that is described in Figure 2. The antibodies can be monoclonal or polyclonal. They can be directed against respectively the entire polypeptide or against fragments thereof. Such an antibody is obtained according to standard methods by immunization of test animals. The antibodies are then used in, e.g., an ELISA (enzyme-linked-immunosorbent assay), in an RIA (radioimmunoassay) or in the immunohistochemistry for determining

the amount of the gene product (Aoki, K. et al. 1996; Forensic Sci. Int. 80, 163-173).

The invention further relates to the use of an antibody chip according to the invention for diagnosis of endometriosis. Antibody chips are miniaturized vehicles, in most cases made of glass or silicon, on whose surface antibodies of known specificity are immobilized in an ordered grid of high density. The detection of the protein/protein interactions can be carried out by mass spectrometry, fluorescence or surface plasmon resonance. Antibodies that specifically bind the proteins that are selected from the group that is described in Figure 2 can be immobilized on the antibody chip. Methods for the production and use of antibody chips are described in Kreider BL, Med Res Rev 2000, 20:212-215.

The mRNA gene products or the cDNA derived therefrom can be determined by hybridization with oligonucleotides, e.g., by a Northern Blot. These oligonucleotides have sequences that are complementary to partial sequences of the gene product that is to be detected and can be labeled with, e.g., a chromogenic, radioactive or fluorescent group. Before hybridization, the cDNA can be amplified with the aid of PCR (Sambrook, J. et al. 1989; Cold Spring Harbor Laboratory Press).

The mRNA gene products or the cDNA derived therefrom can also be determined by quantitative PCR (polymerase chain reaction).

The mRNA can also be determined by **in situ** hybridization with antisense-RNA. In this case, the antisense-RNA can be labeled with dioxigenin, ^{32}P or ^{33}P . Antisense nucleic acid is a DNA and/or RNA, which is complementary to an mRNA. It can comprise the entire complementary sequence or partial sequences. This method is known to one skilled in the art (Barlati, S. et al. 1999; *Histol. Histopathol.* 14, 1231-1240).

The hybridization can also be carried out with the aid of a DNA chip. In addition, the invention therefore relates to a DNA chip, on which at least one oligonucleotide is immobilized, which corresponds to the complete cDNA sequence or a partial sequence or a complementary sequence of a gene that is selected from the group that is described in Figure 1. The invention thus further relates to the use of a DNA chip according to the invention for diagnosis of endometriosis.

DNA chips, also known as DNA microarrays, are miniaturized vehicles, in most cases made of glass or silicon, on whose surface DNA molecules of known sequence are immobilized in an ordered grid of high density. The surface-bonded DNA molecules are hybridized with complementary, optionally labeled nucleic acids. The labeling can be a fluorescence dye.

In the case of oligonucleotide chips, the oligonucleotides that can be bonded to a DNA chip according to the invention represent partial sequences of the gene products (mRNA or cDNA derived therefrom) in the sense or antisense direction. One or more oligonucleotides per gene can be bonded to the DNA chip.

Preferred are 25 nucleotide-long oligonucleotides, which are derived from the non-coding strand. The latter are preferably selected from the respective 3'-untranslated end of the gene. For detection, oligonucleotides of one gene, several genes or all genes that are selected from the group that is described in Figure 1 can be used. Methods for the production and use of DNA chips are described in, e.g., U.S. Patents Nos. 5,578,832, 5,556,752 and 5,510,270.

In the case of cDNA chips, the complete gene products (cDNAs) or subfragments (200-500 bp long) are bonded to the chip. The method is described in, e.g., Eckmann, L. et al., J. Biol. Chem. 2000, 275: 14084-14094.

The mRNA gene product can also be determined by chromogenic assays.

Description of the Figures

Various other features and attendant advantages of the present invention will be more fully appreciated as the same becomes better understood when considered in conjunction with the accompanying drawings, in which like reference characters designate the same or similar parts throughout the several views, and wherein:

Fig. 1 shows the list of genes that can be adjusted downward in the secretory phase in the presence of an endometriosis and thus can be used for a diagnosis of the endometriosis. Listed in column 1 are the names and the data bank number (accession numbers) of the genes, which were found in analysis to be

differentially regulated. In column 2 is found the comparison of samples from the secretory phase (secr. phase), in each case **endometriosis** versus **normal** (no endometriosis); **down** refers to the state of downward adjustment. The first number in parentheses indicates how often the gene was found to be regulated upward, and the second number indicates how often the gene was found to be adjusted downward. For this analysis, 20 individual comparisons were performed. In column 3, the comparison of samples from the proliferative phase (prol. phase) is found. For this analysis, 30 individual comparisons were performed. The designation **down** describes the same state as in column 1, **nc** means **no correlation** (no correlation), i.e., this gene is found to be regulated both downward and upward. The meaning of the numbers is analogous to column 2. In the fourth column, the comparison of samples from the secretory phase with samples from the proliferative phase is found. Here, the endometrium of women without endometriosis was compared to one another. For this analysis, 25 individual comparisons were performed. The designation **up** describes the state of the upward regulation. The meaning of the numbers is analogous to column 2.

Figure 2 shows a list of polypeptides, which are coded by the genes that are depicted in Figure 1 and are expressed to a reduced extent in the presence of an endometriosis.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred

specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

The entire disclosure of U.S. Provisional Application Serial No. 60/243,265, filed October 26, 2000 and German Application No. 100 48 633.9, filed September 25, 2000, are hereby incorporated by reference.

Examples

The molecular-biological methods that are used in the examples, such as, e.g., isolation of RNA, sequencing of DNA, RNase protection, Northern Blot analysis, polymerase-chain reaction (PCR) were performed according to standard protocols, as described in known textbooks, such as in, e.g., Molecular Cloning, A Laboratory Manual (Sambrook, J. et al. 1989; Cold Spring Harbor Laboratory Press). Methods for subtraction analyses of gene expression are described in, e.g., Liang, P. and Pardee, A. B. 1995; Curr. Opin. Immunol. 7, 274-280.

Example 1: Identification of Endometriosis-associated Genes

Genes that are associated with the disease image of endometriosis were identified by comparison of endometrium samples of the following patient groups:

1. Proliferative phase: Days 4-14 after the last menstruation. This group consisted of patients in whom a hysteroscopy or hysterectomy was performed because of leiomyomas.
2. Secretory phase: Days 15-28 after the last menstruation. This group consisted of patients as described under 1.
3. Proliferative phase plus endometriosis: Days 4-14 after the last menstruation. The patients of this group suffered from endometriosis.
4. Secretory phase plus endometriosis: Days 15-28 after the last menstruation. The patients of this group suffered from endometriosis.

The endometrium of women with endometriosis was obtained by means of string curettage. The endometrium of the comparison group was obtained from women within the framework of a hysteroscopy or a hysterectomy, which was performed because of a leiomyoma. The tissue was deep-frozen after removal in liquid nitrogen. Then, whole-RNA was extracted from the samples. This RNA was amplified, labeled by a fluorescence marker, and hybridized with a DNA chip (human SL array of the Affymetrix Company that contains oligonucleotides for about 7000 human genes). After the hybridization process, the DNA chip was

analyzed in a scanner. The hybridization patterns of all gene sequences, which are found on the chip, were compared between all samples. Altogether, 20 individual comparisons with samples from the secretory phase and 30 individual comparisons with samples from the proliferative phase were performed in which in each case a sample was supplied from a woman with endometriosis and a sample from a woman who did not suffer from endometriosis.

All the genes that were adjusted upward or downward in at least half of the cases (10 comparisons) by at least the factor 1.5 relative to the control group (samples of women without endometriosis) were considered to be differentially regulated. In addition, 25 individual comparisons of samples from the secretory phase were performed with samples from the proliferative phase. Here, the endometrium of women without endometriosis was compared.

The results are depicted in Figure 1. The listed genes can be considered as differentiation markers. Assuming that the proliferative phase is dominated by the names according to proliferative processes, the secretory phase is more likely considered as a differentiation phase. Against this background, genes that are important to the differentiation should be regulated upward during this phase (cf. Figure 1, column 4) and regulated downward or regulated to remain at the same level during the proliferative phase (cf. Figure 1, column 3). The genes that are listed in column 1 meet these criteria and are therefore referred to as differentiation markers. The fact that these genes are adjusted downward in women with endometriosis

(column 2) indicates a disrupted differentiation in the secretory phase.

Example 2: Diagnosis of Endometriosis

1. Sample Taking

For the DNA-chip analysis, endometrial tissue is obtained from patients and whole-RNA is isolated therefrom. The RNA is then amplified and coupled to a fluorescence marker. For the immune test, peritoneal fluid, blood, vaginal secretion, urine or endometrial tissue can be obtained from the patient.

2. Detection of Gene Products

2a. Using a DNA Chip

First, the suitable DNA sequences are determined from the genes that are selected from the group that is described in Figure 1. Sequences that can hybridize with the selected gene transcripts are suitable. The oligonucleotides are then produced on the chip by a chemical process that is based on the photolithographic process. To this end, photolithographic masks are used, which were produced by suitable computer algorithms.

The labeled RNA is incubated with the chip in a hybridization furnace. The chip is then analyzed in a scanner, which determines the hybridization profile. As a result, it can be determined whether one or more of the genes of the genes listed in Figure 1 is regulated downward in the secretory phase, which indicates an endometriosis.

2b. By Immune Test

To perform an immune test, specific antibodies that bind to the polypeptides that are described in Figure 2 are required. The antibodies can be monoclonal or polyclonal antibodies, which are directed against the purified proteins, peptides, selected from the coded proteins, or recombinantly produced fragments or whole protein.

If the analysis is carried out by means of immunohistochemistry, the endometrium that was removed from the patient to be analysed is used. After suitable fixing of the tissue, e.g., by means of formaldehyde and subsequent embedding in paraffin, the tissue can be used for the immunohistochemical analysis. To this end, sections of suitable thickness, e.g., 4 μm , are prepared from the fixed and embedded tissue with a microtome. The specific antibody or antibodies are then incubated with the further prepared tissue sections (e.g., process of removing paraffin, blocks) for awhile under suitable temperature conditions, e.g., for one hour at room temperature. After washing steps are carried out with a suitable solution, e.g., PBS, the sections are incubated in a second step with a suitable second antibody that is, e.g., biotinylated for the subsequent reactions. The second antibody binds to the region of the first antibody that is constant for the respective species. After a suitable incubation time and washing steps, the tissue sample is now incubated in a third step with, e.g., horse-radish peroxidase, coupled to streptavidin. After a suitable incubation time and washing steps, an enzyme reaction is now catalyzed in a

last step by adding a suitable dye, e.g., DAB, from the peroxidase, which results in a color reaction where the first antibody specifically bonded. After the enzyme reaction and washing steps are stopped, the tissue section that is dried, fixed and provided with a cover glass can now be analyzed under the microscope. To decide whether a quantitative or else qualitative difference exists in the tissue sample, a corresponding control of a sample from a woman without endometriosis must be used as comparison.

If the analysis is done with Western Blots, the tissue samples or extracts that are obtained are separated from the peritoneal fluid, blood, vaginal secretion or urine by means of a polyacrylamide-gel electrophoresis. After the separation, the polypeptides that are separated in the gel are moved to a suitable carrier membrane, e.g., nitrocellulose, by application of an electric current. The proteins that are fixed to the carrier membrane are now incubated in a first step with the specific antibody or antibodies. After suitable washing processes with, e.g., TBS/TBST, the carrier membrane is incubated in a second step with a second antibody, which binds to the region of the first antibody that is constant for the respective species. The second antibody can carry a radioactive labelling or a coupled enzyme, e.g., alkaline phosphatase, which converts a colorless substrate into a colored substrate in a subsequent color reaction. Since the amount of the antigen is proportional to that of the second antibody that is bonded to the antigen, the amount of the measured dye can therefore be used for a

quantitative analysis of the polypeptide or polypeptides that are present in the extract.

If the analysis is done with a solid-phase immunoassay, the specific antibody or antibodies are bonded to a polymer carrier matrix, e.g., polyvinyl chloride. The fixed antibody or antibodies are then incubated with the extract, which was obtained from, e.g., the peritoneal fluid, blood, vaginal secretion or urine. After suitable washing processes, a second antibody that specifically binds to another site of the antigen that is to be detected is added in a second step. The second antibody carries, e.g., a radioactive or fluorescence labeling and can therefore be detected in a highly sensitive manner in a third step. The amount of the second antibody that is bonded to the antigen is proportional to that of the antigen and can therefore be used for a quantitative analysis of the protein or proteins that are present in the extract.

If the analysis is done by means of ELISA (enzyme linked immunosorbent assay), the specific antibody or antibodies are bonded to a polymer carrier matrix, e.g., polyvinyl chloride. The fixed antibody or antibodies are then incubated with the extract, which was obtained from, e.g., the peritoneal fluid, blood, vaginal secretion or urine. After suitable washing processes, a second antibody is added in a second step, and said antibody specifically binds to another site of the antigen that is to be detected. In addition, the second antibody carries a coupled enzyme, e.g., an alkaline phosphatase. This enzyme now catalyzes in a subsequent step the conversion of a colorless

substrate into a colored product. A non-fluorescent substrate can also be converted into a fluorescent substrate, however. The amount of colored or fluorescent product can be measured colorimetrically. Since the amount of the second antibody that is bonded to the antigen is proportional to that of the antigen, the amount of the measured dye or fluorescence product can therefore be used for a quantitative analysis of the polypeptide or polypeptides that are present in the extract.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.